⑧ & ⑨ Atopic dermatitis, Contact dermatitis

Original Paper

International Archives of Allergy and Immunology

Int Arch Allergy Immunol 2000;123:341–348

Received: May 16, 2000
Accepted after revision: August 29, 2000

Suppressive Effect of Topically Applied CX-659S, a Novel Diaminouracil Derivative, on the Contact Hypersensitivity Reaction in Various Animal Models

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Key Words
Contact hypersensitivity reaction · Picryl chloride · Oxazolone · 2,4-Dinitrochlorobenzene · CX-659S · Uracil derivatives · IL-1β · TNF-α

Abstract
Background: The T-cell-mediated contact hypersensitivity reaction (CHR) is thought to be involved in the pathogenesis of clinical cutaneous disorders including atopic dermatitis. A novel diaminouracil derivative, CX-659S, has been reported to have an inhibitory activity against picryl chloride (PC)-induced CHR when administered either orally or percutaneously. The inhibitory effect of topical CX-659S was assessed in three CHR models in the present study. In addition, to elucidate the mechanism of action of this compound, we examined the effect of CX-659S on the expression of messenger RNAs for proinflammatory cytokines after elicitation in PC models. ***Methods:*** For the in vivo evaluation of the efficacy of CX-659S, we used PC- or oxazolone-induced CHR in mice and 2,4-dinitrochlorobenzene (DNCB)-induced CHR in guinea pigs. CX-659S was topically applied immediately after the hapten challenge in each model. To assess the effect on gene expression of cytokines, we used the reverse transcriptase-polymerase chain reaction (RT-PCR), a semiquantitative technique with specific primers. ***Results:*** Topical CX-659S dose-dependently inhibited ear swelling at 24 h after the challenge in the two mouse models. This inhibitory effect was histologically confirmed in the PC model. Topically applied CX-659S also inhibited erythema and edema formation 24 h after challenge in the guinea pig model. CX-659S inhibited the expression of mRNA for proinflammatory cytokines IL-1β and TNF-α in vivo. ***Conclusions:*** Topically applied CX-659S showed significant inhibitory activities against CHR models both in mice and in guinea pigs. Inhibition profiles of CX-659S toward mRNA expression for proinflammatory cytokines corroborated these findings. CX-659S thus could be a useful therapeutic agent for allergic cutaneous disorders such as allergic contact dermatitis and atopic dermatitis.

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Introduction

The contact hypersensitivity reaction (CHR) is regarded as a prototype of T-cell-mediated delayed-type hypersensitivity reactions. Since Landsteiner and Jacobs [1] first reported the CHR model in guinea pigs, there have been numerous reports on CHR animal models using various chemical haptens, such as picryl chloride (PC), 2,4-dinitrochlorobenzene (DNCB) and oxazolone in mice and in guinea pigs. According to the current knowledge [2, 3], during the afferent or sensitization phase, epicutaneously applied hapten is taken up and processed by epidermal Langerhans cells (LCs). LCs then migrate to the draining lymph nodes and present hapten-major histocompatibility complexes (MHC) to naive T cells, which are then converted to hapten-specific T cells. In the efferent or elicitation phase, reapplication of the same hapten results in the recruitment of hapten-specific T cells, which react to the hapten presented by LCs, release cytokines and chemokines, and attract other inflammatory cells. As a result, cutaneous swelling and edema occur. Numerous studies have revealed that multiple cell types, chemical mediators, cytokines and chemokines are involved in the regulation of these processes in CHR [4, 5]. Among these, the proinflammatory cytokines IL-1β and TNF-α have been reported to have essential roles in the initiation and progression of these processes [6, 7].

The CHR in animals is a reliable and reproducible model for clinical allergic contact dermatitis. In addition, T cells are thought to play a major role in the pathogenesis of other clinical cutaneous disorders such as atopic dermatitis [8]. Therefore, compounds that have an inhibitory activity on the CHR are thought to be useful not only as a tool for elucidating the mechanism of this reaction in animal models but also as a drug for the treatment of allergic contact dermatitis and other T-cell-mediated skin disorders including atopic dermatitis in humans.

We have recently reported that in the course of screening for compounds that block the PC-induced CHR in mice, we found a novel diaminouracil derivative, CX-659S [(*S*)-6-amino-5-(6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxamido)-3-methyl-1-phenyl-2,4(1*H*,3*H*)-pyrimidinedione] (fig. 1), to have inhibitory activity against this reaction by both oral and percutaneous administration [9].

In this study, to ascertain the potency of CX-659S as a topical anti-allergic agent, we extensively investigated the inhibitory effects of topical CX-659S on two CHR models in mice and on one in guinea pigs. In addition, to elucidate the mechanism of this compound, we examined the effect of CX-659S on the expression of messenger RNAs for proinflammatory cytokines, such as IL-1β and TNF-α, after elicitation in PC models by using the semiquantitative reverse transcriptase-coupled polymerase chain reaction (RT-PCR) technique.

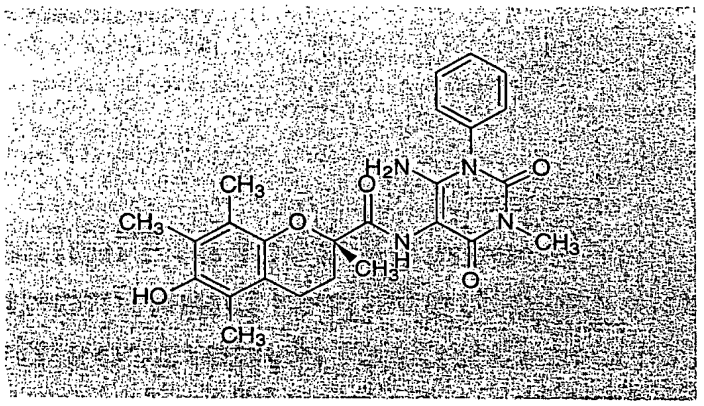


Fig. 1. Chemical structure of CX-659S (MW: 464.5).

Materials and Methods

Animals

6-week-old male ICR mice were purchased from Clea Japan, Inc. (Tokyo), and 5-week-old male Hartley guinea pigs, from Japan SLC, Inc. (Hamamatsu, Japan). The animals were maintained on a 12-hour light/12-hour dark cycle, and the room temperature was set at 23 °C. They were fed food and tap water ad libitum. The study protocol was approved by the Japan Energy Animal Care and Use Committee.

Drugs

PC and DNCB were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo). 4-Ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxazolone) was obtained from Aldrich Chemical Co. (Milwaukee, Wisc., USA). Betamethasone 17-valerate was purchased from Sigma Chemical Co. (St. Louis, Mo., USA).

PC-Induced CHR in Mice

Sensitization and Challenge. Eight mice in a group were sensitized by applying 0.1 ml of 7% PC solution in acetone to the shaved abdomen. The nonsensitized group was shaved but not sensitized. Each mouse was kept separately in a stainless steel wire-mesh cage to avoid contact. Seven days later, the mice were challenged by applying 0.02 ml of 1% PC solution in acetone to the left ear (0.01 ml each on the dorsal and ventral side). The ear thickness was measured with a calibrated digital thickness gauge (Mitutoyo Corp., Tokyo) before and 24 h after the challenge, and the difference in thickness was calculated. After the 24-hour measurement, the left ears were excised for

histological evaluation. Test compounds in acetone or vehicle in a volume of 0.04 ml were painted on the left ear immediately after the challenge.

Histological Analysis. The ears were fixed in 10% neutral-buffered formalin, dehydrated, and embedded in paraffin by standard procedures. Sections were stained with hematoxylin and eosin. Histological examination was made by light microscopy. The numbers of neutrophils and eosinophils were counted in high-power fields (HPF; × 400) and expressed as total cell numbers in five alternate HPFs (the 2nd, 4th, 6th, 8th and 10th HPF from the edge of each ear).

Oxazolone-Induced CHR in Mice

For sensitization and elicitation, 0.5% oxazolone solution in ethanol and in acetone, respectively, was used. Other experimental conditions were the same as those for PC as mentioned above. Test compounds in acetone: AcOEt (99:1 v/v) or vehicle in a volume of 0.04 ml were painted on the left ear immediately after the challenge.

DNCB-Induced CHR in Guinea Pigs

Six guinea pigs in a group were sensitized by applying 0.01 ml of 39% DNCB solution in acetone to the atrichous area of the root of each ear. Fourteen days later, the animals were challenged by applying 0.01 ml of 0.5% DNCB solution in ethanol to the shaved backs. Test compounds in acetone in a volume of 0.04 ml were applied to the sites 5 min after the challenge. Vehicle (acetone) in a volume of 0.04 ml was applied to the control site. Six sites per animal at intervals of about 2.5 cm were used, and the positions of each test site were different in each animal. Sites were examined for erythema and edema at 24, 48 and 72 h after the challenge. Erythema and edema were graded on a scale of 0 to 5, where a score of 0 indicated no reaction, a score of 2 indicated a pale pink color and no edema, and a score of 5 represented a bright red color and edema.

Analysis of Cytokine mRNA Expression in Mouse Ears

Treatment of Mice and RNA Extraction. Four to five mice in a group were sensitized by applying 0.1 ml of 7% PC solution in acetone to the shaved abdomen. Seven days later, the mice were challenged by applying 0.02 ml of 1% PC solution in acetone to the left ear. On the mice in one group (elicitation (–)), 0.02 ml of vehicle (acetone) was painted. Test compounds in acetone or vehicle in a volume of 0.04 ml were then painted on the left ear immediately after the challenge. After 2 h, the mice were sacrificed by CO_2 inhalation, and the left ears were removed. Total RNA was extracted from the ear of each mouse by using an RNeasy® Total RNA kit (Qiagen Inc., Chatsworth, Calif., USA) according to the manufacturer's protocol. The extracted RNA was suspended in 50 µl of diethylpyrocarbonate-treated water, and its concentration was determined spectrophotometrically at a wavelength of 260 nm.

RT-PCR. A portion (500 ng) of the total RNA prepared from the left ear of each mouse was reverse-transcribed in a reaction mixture (20 µl) consisting of 1 µ*M* $pd(T)_{12-18}$, 20 units RNasin® ribonuclease inhibitor (Promega), 0.5 m*M* concentration of each dNTP (Pharmacia), and 200 units of Moloney murine leukemia virus reverse transcriptase (Life Technologies Inc.). The reaction mixture was incubated at 42 °C for 1 h, and then heated at 95 °C for 3 min to inactivate the enzyme. Each gene of interest was then amplified by PCR using the resulting cDNA as a template, in a reaction mixture containing dNTPs, *Taq* polymerase, [α-^{32}P]dCTP (~ 110 TBq/mmol, Amersham) and 0.2 µ*M* of each specific primer in a total volume of 50 µl. Primers used were 5′-ATG GCA ACT GTT CCT GAA CTC AAC T-3′ and 5′-CA GGA CAG GTA TAG ATT CTT TCC TTT-3′ for IL-1β; 5′-TTC TGT CTA CTG AAC TTC GGG GTG ATC GGT CC-3′ and 5′-GTA TGA GAT AGC AAA TCG GCT GAC GGT GTG GG-3′ for TNF-α; and 5′-GTG GGC CGC TCT AGG CAC CAA-3′ and 5′-CTC TTT GAT GTC ACG CAC GAT TTC-3′ for β-actin. After an initial incubation at 95 °C for 1 min, PCR was run for 30 cycles under the following conditions: denaturation at 95 °C for 30 s, annealing at 55 °C for 40 s, and extension at 72 °C for 1 min. It should be noted that the amounts of the products amplified under this condition were proportional with respect to the amounts of cDNA added to the reaction.

Semiquantitative Analysis of PCR Products. Amplified products were separated by electrophoresis on 4% polyacrylamide gel, and the amount of the product with the expected size was determined by using a bio-image analyzer BAS2000 (Fuji Photo Film Co., Ltd.). For relative semiquantitation, we calculated the relative mRNA levels by dividing the intensities of the product for each cytokine by those of a housekeeping gene, β-actin.

Statistics

Results are expressed as the mean ± SEM. Comparisons among the groups were performed by Dunnett's test. $p < 0.05$ was considered to be statistically significant.

Results

Effect of CX-659S on PC-Induced CHR in Mice

Figure 2 shows the effect of topical CX-659S on the PC-induced CHR in mice. The topical CX-659S applied immediately after the challenge inhibited ear swelling dose-dependently. The inhibitory effect of CX-659S at a dose of 0.1 mg/ear or more was statistically significant. Betamethasone 17-valerate at a dose of 0.01 mg/ear also strongly inhibited ear swelling in this model.

The effect of CX-659S was further investigated histologically. The ear of each mouse used in the above experiment was removed after the thickness measurement, fixed, and stained with hematoxylin and eosin. Figure 3 shows representative results for samples of ears from unsensitized mice (fig. 3a) or mice sensitized, challenged, and treated with either vehicle (acetone, fig. 3b), CX-659S (1.0 mg/ear, fig. 3c) or betamethasone 17-valerate (0.01 mg/ear, fig. 3d). Severe edema and marked inflammatory cell infiltration were seen in the ear challenged and treated with the vehicle control. However, in the ear treated with either CX-659S or betamethasone 17-valerate, reduced edema and diminished inflammatory cell infiltration were observed. We additionally counted the numbers of neutrophils and eosinophils infiltrating the epidermis and dermis. As indicated in table 1, the numbers of these inflammatory cells in the ear of unsensitized but challenged mice were considerably increased, presumably

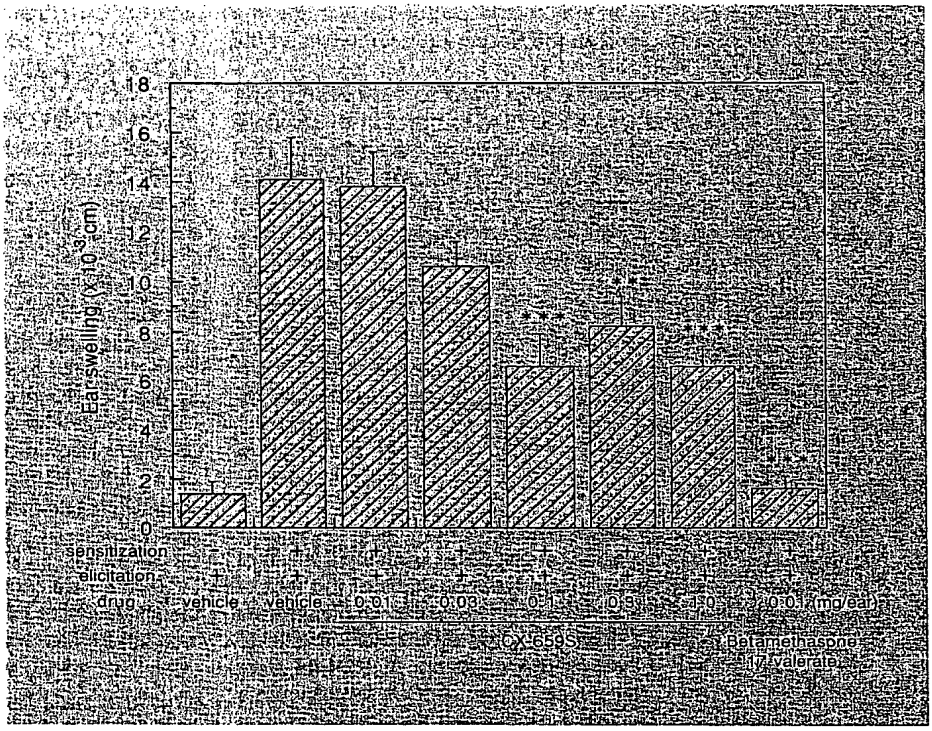
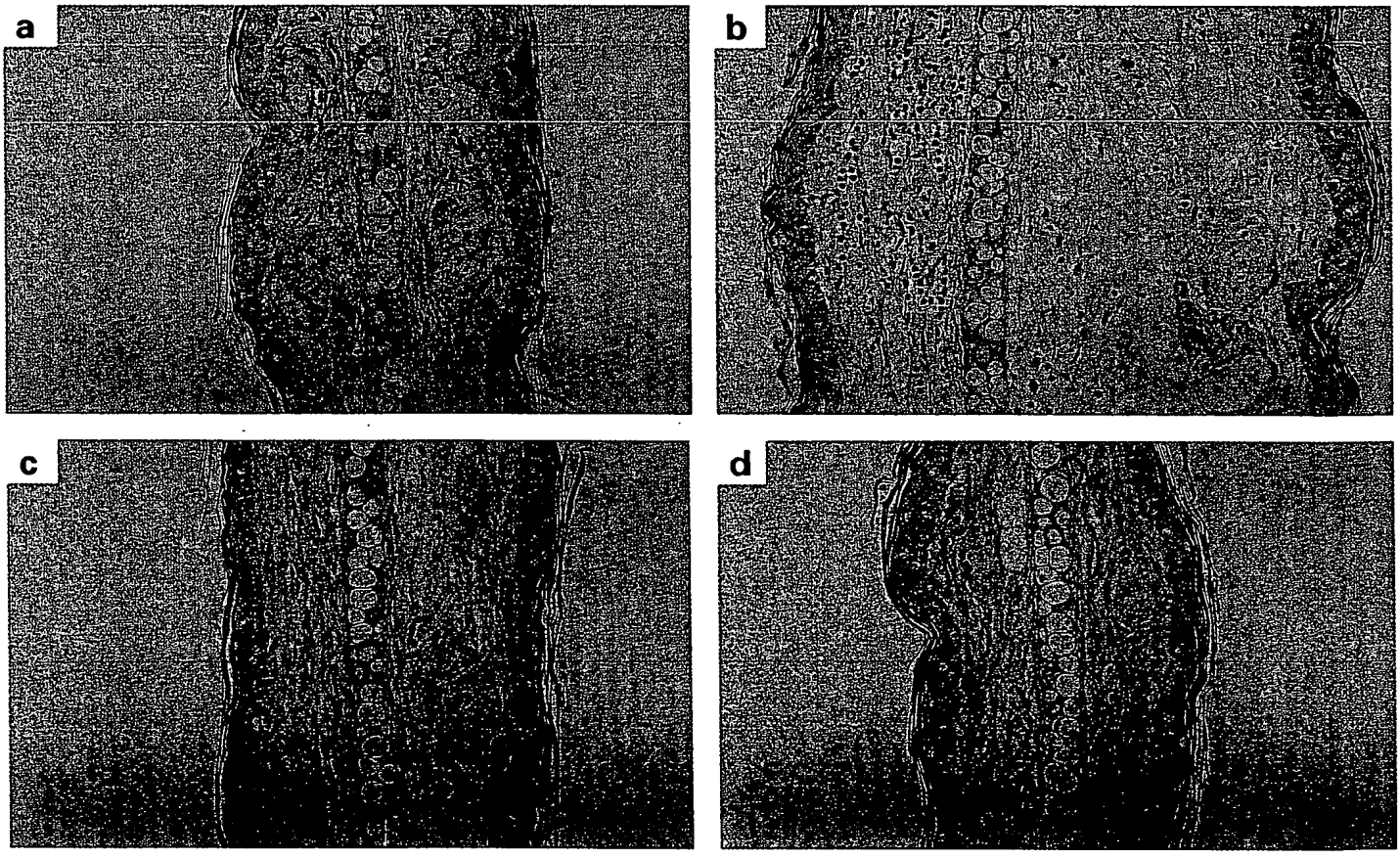


Fig. 2. Effect of CX-659S on PC-induced CHR in mice. Sensitized ICR mice were challenged by applying 0.02 ml of 1% PC solution in acetone to the left ear. Compounds were painted on the ear immediately after the challenge. The ear thickness was measured before and 24 h after the challenge, and the difference in thickness was calculated. The results are expressed as mean ± SEM of 8 mice. ** $p < 0.01$; *** $p < 0.001$ vs. control.

Fig. 3. Histological appearance of ears with PC-induced CHR. The ears obtained from the experiment in figure 2 were fixed in 10% neutral-buffered formalin, dehydrated, and embedded in paraffin. Sections were stained with hematoxylin and eosin. Histological examination of ears unsensitized (**a**), or of those treated with either vehicle (acetone) (**b**), CX-659S (1.0 mg/ear) (**c**) or betamethosone 17-valerate (0.01 mg/ear) (**d**) was done by light microscopy. × 308.



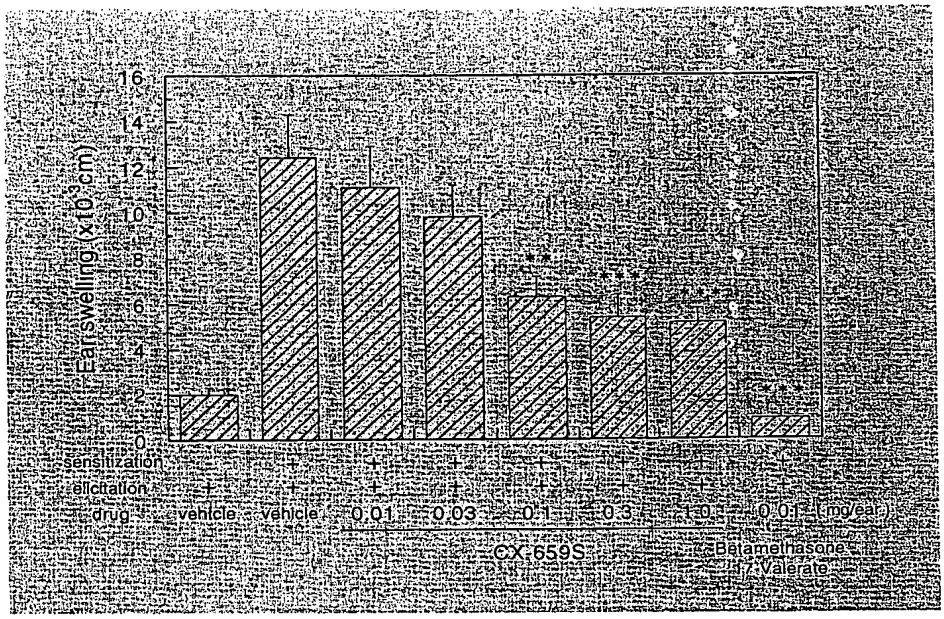


Fig. 4. Effect of CX-659S on oxazolone-induced CHR in mice. Sensitized ICR mice were challenged by applying 0.02 ml of 0.5% oxazolone solution in acetone to the left ear. Compounds were painted on the ear immediately after the challenge. The ear thickness was measured before and 24 h after the challenge, and the difference in thickness was calculated. The results are expressed as mean ± SEM of 8 mice. ** $p < 0.01$; *** $p < 0.001$ vs. control.

Table 1. Effect of CX-659S on inflammatory cell numbers infiltrating skin lesions 24 h after challenge in PC model

Treatment		Drug	Dose	Neutrophil	Eosinophil
sensitization	challenge		mg/ear		
–	–	–		0	6.5
–	+	vehicle		127.5 ± 31.5[a]	28.8 ± 9.7
+	+	vehicle		421.8 ± 117.8	74.4 ± 25.8
+	+	CX-659S	0.01	295.6 ± 79.4	54.8 ± 12.8
+	+	CX-659S	0.03	305.9 ± 62.3	64.1 ± 11.3
+	+	CX-659S	0.1	104.3 ± 41.7**	17.9 ± 5.6*
+	+	CX-659S	0.3	190.1 ± 37.6*	46.1 ± 8.9
+	+	CX-659S	1.0	64.9 ± 12.7***	20.0 ± 5.5*
+	+	betamethasone	0.01	15.3 ± 3.0***	17.6 ± 5.6*

[a] Mean ± SEM. Values are total cell numbers in 5 HPFs (400 ×). Ear samples were obtained 24 h after the PC challenge and drug painting. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control.

because of a non-hapten-specific inflammatory process induced by PC painting. The inflammatory cell numbers were more increased in the ear of mice sensitized, challenged, and treated with vehicle. CX-659S treatment at doses of 0.1 mg/ear or more decreased the numbers of both neutrophils and eosinophils. Betamethasone 17-valerate treatment at a dose of 0.01 mg/ear also significantly decreased the numbers of both cell types.

Effect of CX-659S on Oxazolone-Induced CHR in Mice

The effect of CX-659S on the CHR induced by oxazolone, another well-evaluated hapten, was examined in mice. As shown in figure 4, topical CX-659S applied immediately after the challenge inhibited ear swelling in a dose-dependent manner. The effect of CX-659S at doses of 0.1 mg/ear or more was statistically significant. Betamethasone 17-valerate at a dose of 0.01 mg/ear also strongly inhibited ear swelling in this model.

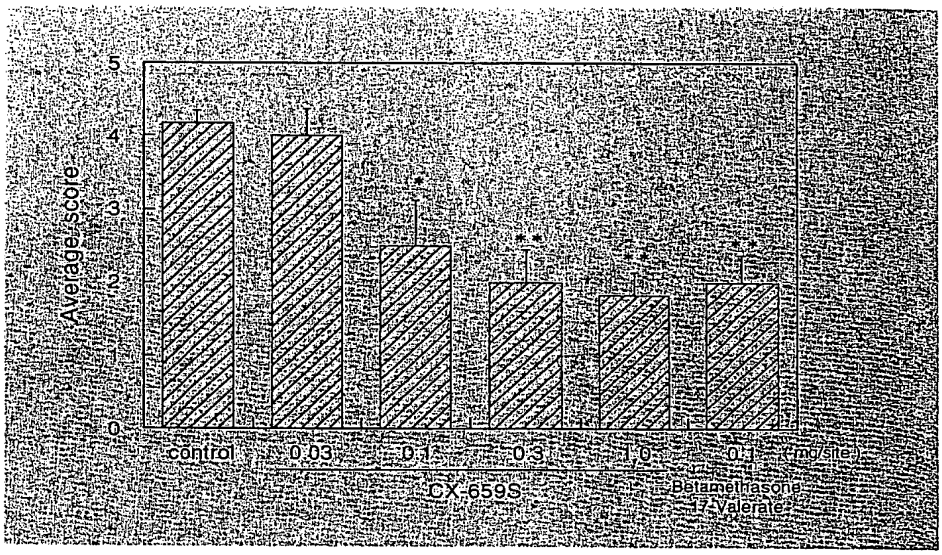


Fig. 5. Effect of CX-659S on DNCB-induced CHR in guinea pigs. Sensitized guinea pigs were challenged by applying 0.01 ml of 0.5% DNCB solution in ethanol to the shaved backs. Compounds in acetone in a volume of 0.04 ml were applied to the sites 5 min after the challenge. Sites were examined for erythema and edema at 24 h after the challenge. Erythema and edema were graded on a scale of 0–5. * $p < 0.05$, ** $p < 0.01$ vs. control.

Effect of CX-659S on DNCB-Induced CHR in Guinea Pigs

We next examined the effect of topical CX-659S on the CHR in guinea pigs. As shown in figure 5, topical CX-659S applied 5 min after the challenge dose-dependently suppressed the erythema and edema formation at 24 h after the challenge. The effect of CX-659S at a dose of 0.1 mg/site or more was statistically significant, and the average scores at doses of 0.3–1.0 mg/site were comparable to the score for betamethasone 17-valerate at a dose of 0.1 mg/site. At 48 or 72 h after the challenge, CX-659S also suppressed the reaction, but the effect was not statistically significant then. Betamethasone 17-valerate also suppressed the reaction at 48 or 72 h (statistically significant at 48 h) (data not shown). Although the suppressive effect of CX-659S was also seen when the drug was applied 15 min before or after the challenge, the effect was not observed when CX-659S was applied 60 min after the challenge. Betamethasone 17-valerate was not effective when applied 15 min before or after and 60 min after the challenge (data not shown).

Effect of CX-659S on Cytokine mRNA Expression in Mouse Ears

As an approach to investigate the mechanism of the inhibitory activity of CX-659S against CHR, we examined the effect of topical CX-659S on the mRNA expression of two proinflammatory cytokines, IL-1β and TNF-α, after hapten challenge in the PC-induced CHR model in mice. Firstly, time-course experiments showed that both IL-1β and TNF-α mRNA levels were upregulated within 1 h and reached their peak levels at 2–4 h after the PC challenge (data not shown), confirming the findings of Enk and Katz [10]. Therefore we decided to measure the mRNA levels of these cytokines in the ear of mice 2 h after the challenge. As shown in figure 6, both IL-1β and TNF-α mRNA levels were significantly increased in the PC-treated and acetone-painted ear. CX-659S treatment decreased mRNA levels for these cytokines in a dose-dependent manner. The effect of CX-659S at doses of 0.1 mg/site or more was statistically significant, in good agreement with its effect on the ear-swelling response (fig. 2). Betamethasone 17-valerate at a dose of 0.01 mg/ear also significantly inhibited the expression of mRNA for these cytokines. Decreased expression of mRNA for IL-1β and TNF-α was also seen in the ears of oxazolone-treated mice painted with CX-659S (data not shown).

Discussion

The present study demonstrated that topically applied CX-659S inhibited the ear swelling in PC-induced CHR in mice (fig. 2) and that CX-659S reduced the number of inflammatory cells infiltrating into the epidermis and dermis (fig. 3, table 1). CX-659S also inhibited the oxazolone-induced CHR in mice (fig. 4) and the DNCB-induced CHR in guinea pigs (fig. 5). These results indicate that CX-659S has general inhibitory activities against

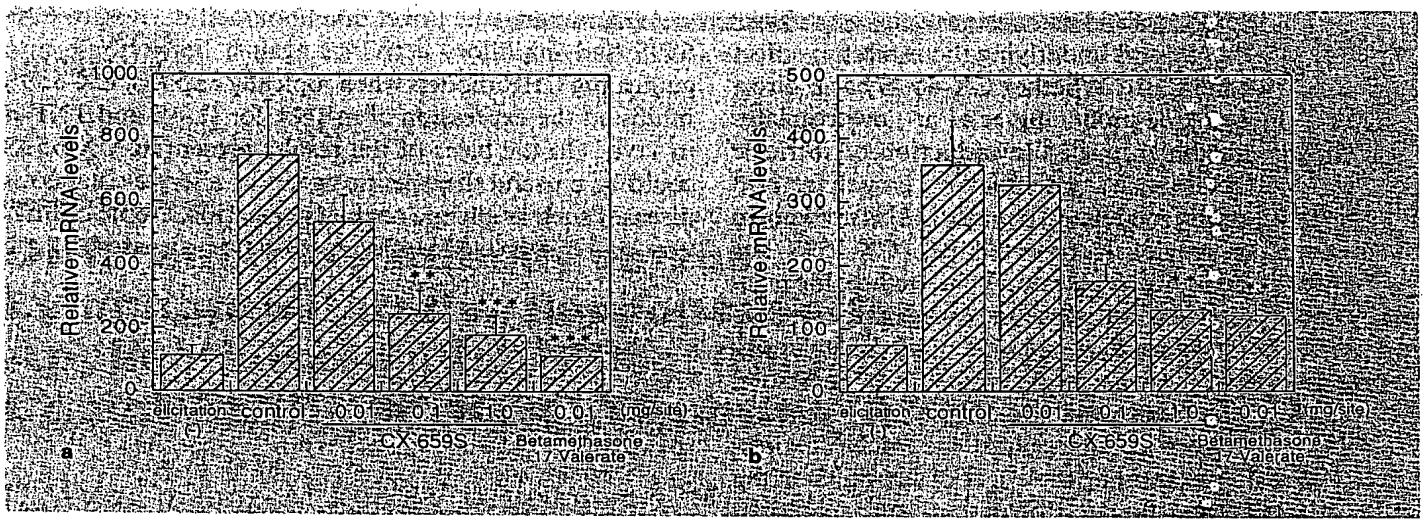


Fig. 6. Effect of CX-659S on mRNA expression of IL-1β (**a**) and TNF-α (**b**) in PC-induced CHR. Sensitized mice were challenged by applying 0.02 ml of 1% PC solution in acetone to the left ear. The mice in the elicitation (–) group were not challenged, vehicle was applied instead. Test compounds in acetone or vehicle were painted on the left ear immediately after the challenge. Total RNA was extracted from the painted ear at 2 h after the challenge. The RT-PCR was performed by using specific primers for IL-1β or TNF-α. The intensities of the product for each cytokine were normalized to those of the housekeeping gene, β-actin. The results are expressed as mean ± SEM of 4 or 5 mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control.

erythema and edema formation in CHR independent of the nature of haptens or animal species. It is to be noted that in the guinea pig model, the inhibitory activity of CX-659S at a dose of 0.3 mg/site or more was comparable to that of betamethasone 17-valerate. The structure and barrier properties of the skin in man are well known to resemble those in guinea pigs more than those in mice. In addition, guinea pigs are reported to be relatively resistant to steroids [11], and, except for steroids and some immunosuppressive agents such as cyclosporin A [12, 13] and FK-506 [14], few compounds are known to have suppressive effects against CHR in guinea pigs by topical application [15]. Taken together, the data would suggest CX-659S to be an effective topical drug for the treatment of cutaneous disorders in humans.

As concerns the toxicological aspects, topical steroids such as betamethasone 17-valerate are well known to have several side effects including atrophia cutis which may be involved, in part, in the decrease of ear thickness with the treatment of these types of compounds. While, in our preliminary toxicological studies, topical CX-659S was shown to produce no symptom of atrophia cutis, and the results on several cutaneous toxicity tests such as skin sensitization test and skin photosensitization test were negative (data not shown). These results suggest that CX-659S is expected to be a low-toxicity compound.

For the mechanism of the inhibitory action toward the CHR, we reported in this paper that CX-659S inhibited the mRNA expression of two proinflammatory cytokines, IL-1β and TNF-α, after challenge of mice in which a PC- or oxazolone-induced CHR occurred. The LC-derived IL-1β is thought to play an important role in the initiation of immune responses in the CHR [6]. IL-1β mRNA is induced in the skin within 15 min after exposure to hapten [10], and injection of IL-1β protein induces other cytokine mRNA changes, mimicking those caused by haptens [6] and enhances MHC class II expression in LCs [6]. Furthermore, administration of anti-IL-1β antibody [6] or IL-1 receptor antagonist [16] is reported to suppress the CHR in mice. An experiment by Piguet et al. [7] showed that TNF-α is also a critical mediator of the CHR in mice. TNF-α mRNA was increased within 30 min after hapten challenge, and anti-TNF-α antibody abrogated the ear swelling response as a measure of the CHR, when administered immediately before the challenge [7]. Therefore, we may speculate that the inhibitory activity of

CX-659S toward the mRNA expression of these cytokines is one of the mechanisms underlying the inhibitory effect of the drug on the CHR. Whether CX-659S has a direct effect on the cytokine-producing cells or downregulates cytokine production through the induction of intrinsic inhibitory molecules such as transforming growth factor-β [17] and calcitonin gene-related peptide [18] is not known at present. As previously reported [9], CX-659S has antioxidative activities as assessed by a method for measuring lipid peroxidation. Reactive oxygen species are known to be involved in the regulation of intracellular signaling pathways for gene expression [19, 20]. Therefore, the antioxidative properties of CX-659S may be responsible for the inhibitory activity against the mRNA expression. We are now examining the effect of CX-659S on the gene expression and function of dermal and epidermal cells in detail with ex vivo and in vitro techniques.

In summary, the novel diaminouracil derivative CX-659S had inhibitory activities against both mouse and guinea pig CHR models when topically applied. CX-659S inhibited the expression of mRNA for IL-1β and TNF-α in vivo. On the basis of the results presented, CX-659S could be a useful therapeutic agent for allergic cutaneous diseases such as allergic contact dermatitis, atopic dermatitis, and maybe other inflammatory skin diseases.

Acknowledgements

We wish to thank K. Katoku of Panapharm Laboratories Co., Ltd., for histological analysis and M. Mizunuma for her excellent technical assistance.

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